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A STUDY OF THE DRY HEAT RESISTANCE OF NATURALLY OCCURRING ORGANISMS WIDELY DISPERSED ON A SURFACE

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Abstract

Although Bacillus subtilis var. niger is the standard test organism for NASA Planetary Quarantine sterilization studies, it has been found that some naturally occurring soil organisms are more heat resistant. This report describes the separation of the heat resistant organisms from soil particles and the experiments designed to show that the heat resistance is a natural characteristic of the organisms rather than a condition induced by the clumping effect of agglomerated particles and organisms.

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Introduction

Until recently, most of the sterilization experimentation associated with the NASA Planetary Quarantine Program concerned itself with the standard test organism, _Bacillus subtilis_ var. _niger_. This organism, which is non-pathogenic and lends itself to laboratory experimentation, is considered to be relatively resistant to dry heat inactivation. For this reason, it was chosen as a representative candidate for dry heat sterilization studies(1).

Subsequent work by the Phoenix Laboratories, USPHS(2), and Sandia Laboratories(3) indicated that some of the naturally occurring soil organisms from Cape Kennedy displayed a much greater D-value* than _Bacillus subtilis_ var. _niger_. Further, it was shown that these soil organisms can be transported into buildings at Cape Kennedy in which spacecraft are assembled and tested(3), where their presence could extend spacecraft sterilization requirements drastically.

The object of the series of experiments described here was to determine whether the higher heat resistance of these naturally occurring spores was induced through protection afforded by clumping with soil or other spores. In these experiments, we separated spores from the soil particles and attempted to disperse the organisms to about 40 per square foot on the test surface area. In past experiments with Cape Kennedy soil, no effort was made to minimize clumping to this extent. Therefore, clumping conditions much beyond those normally found on spacecraft surfaces could have existed.

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*D-value is defined as the time required to reduce a given population of viable organisms by one log or 90% at a specified temperature.

Materials and Methods

Preparation of Spore Stock

The stock used for these experiments was obtained by putting 5 grams of Cape Kennedy soil through a series of Freon TF washing operations. About one liter of the Cape soil was obtained by the Spacecraft Bioassay Unit, PHS, Phoenix, from several locations outside and adjacent to the AO Building at Cape Kennedy. After the larger pieces of debris were removed, the soil was sieved to a size of less than 147 microns. The entire amount of dust was then tumbled for 11 hours to mix it thoroughly and to provide a homogeneous sample stock.

The liquid selected as the dissociation medium was Freon TF. This selection was made for the following reasons:

a. The density of Freon TF is 1.55, whereas the density of spores is approximately 1.2 - 1.3. It was believed that this difference in physical properties would be beneficial in floating spores to the top of any Freon/dust solution.

b. The viscosity of Freon TF is 0.682 centipoises at 25°C. The viscosity of other candidate liquids at the same temperature is 0.894 for water and about 1.265 for 95% ethanol. This property facilitates both separation and filtering.

c. The solvent properties of Freon TF dissolve any oily films present which act as a binder between the particles and organisms.

d. Freon TF exhibited no noticeable sporostatic effects on these organisms[3].

e. Freon TF is readily available commercially.

An important feature of the stock preparation was the preconditioning of the dry soil particles prior to Freon washing. Due to the extreme hydrophobic quality of Freon and the desire to work only with the more heat resistant organisms, the soil sample was heated in an evacuated oven for 1.5 hours at 125°C to dry the soil and inactivate the less heat resistant organisms. At the end of this period, the oven was back-filled with dry nitrogen (N_2) prior to opening the door. The loosely capped bottle containing the soil was then closed tightly, removed from the oven, and allowed to cool to room temperature. Freon was added immediately upon removal of the cap. Thus ambient air was not permitted to contact the soil before the addition of Freon.

Each wash operation consisted of adding about 30 ml of Freon to the dust, insonating the mixture for 30 seconds, allowing the particles to settle, and drawing off the supernatant into a sterile beaker. This procedure was repeated eight times. The collected supernatant was then subjected to another series of eight similar washing operations. The supernatant from the second series of washes was filtered through a 0.8μ filter and the filter was insonated for about 30 seconds in 20 ml of ethanol. This solution was strained through a sterile, 300 mesh sieve to remove filter particles and an additional 30 ml of ethanol were added to constitute our base stock of about 300 organisms per ml.

Preparation and Exposure of Samples

A working stock was prepared by diluting an appropriate amount of the base stock with ethanol. Our target was about eight organisms per ml. One ml of the new stock was pipetted into each 150 mm glass Petri dish, which represents an area of ~0.2 square foot. The plates were then rotated in such a manner that the solution spread over the entire surface. After the excess ethanol had evaporated, the plates were placed in a recirculating oven at 125°C and removed at hourly intervals up to eight hours. Each hourly sample consisted of three plates which represented about 3/5 of a square foot. After cooling to room temperature, the plates were overlayed twice with Trypticase Soy Agar with 0.1% soluble starch and 0.2% yeast extract added. The plates were incubated at 32°C and counted after 10 days incubation.

Other stocks were prepared using different dilutions of the base stock in order to produce different surface bioburden loadings. In these additional tests, samples were removed from the oven at two hour intervals up to a total of eight hours.

Results

Even with what appeared to be appropriate dilution of the original stock, we found it difficult to attain a precise population of 40 organisms per square foot. In the first experiment, we had an average of 16 organisms per square foot. While this presented the situation of working with low numbers of colonies per plate, it also reduced the possibility of affording clumping protection to the organisms. In the second and third experiments, the average microbial loading was approximately 11 and 43 organisms per square foot, respectively.

The density and size of soil particles present on the plates are shown in Figures 1 and 2. Figure 1 represents a heavy loading for this experiment and Figure 2 a light loading. Each division on the attached scales represents 10μ. These photomicrographs show actual dispersion of particles and indicate the lack of any particle clumping.

When working with extremely low numbers, there is always the possibility of biasing the results due to the probability of any one sample not being representative of the entire working stock. However, this was one of the constraints imposed on these experiments in order to approximate what is thought to be a more realistic loading of the heat resistant organisms. The consistency and repeatability of the data, as shown in Figure 3 for the first experiment, indicates that low numbers were not a problem and that a high degree of validity was attained. The raw data for the other two experiments were similarly consistent.

The estimated D-values at 125°C for all three experiments were approximately 35-40 hours (Figure 4), where an extremely sparse population

of organisms was present. This compares to the 125°C D-value for Bacillus subtilis var. niger of 25-30 minutes.

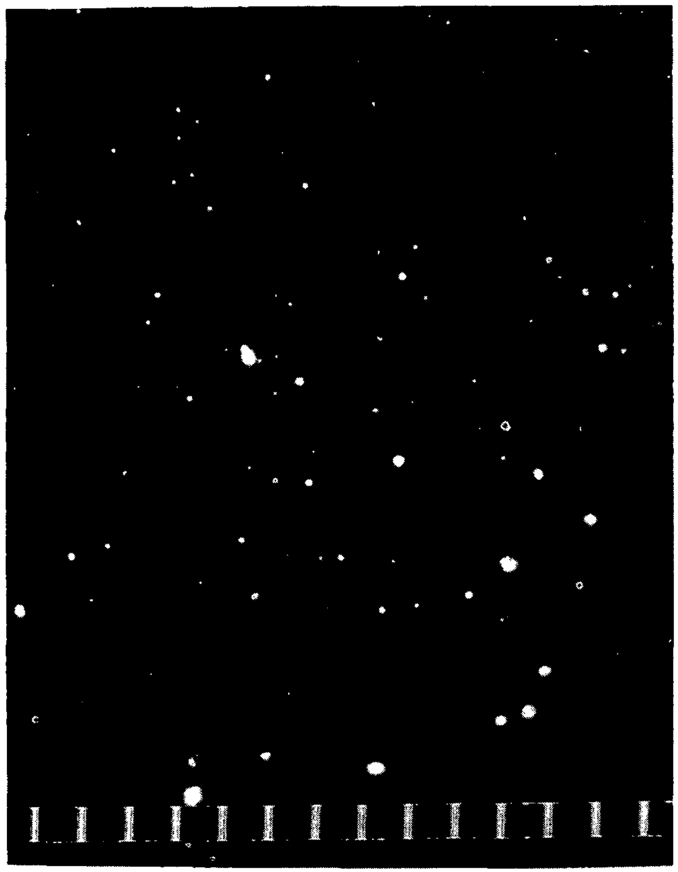

Figure 1

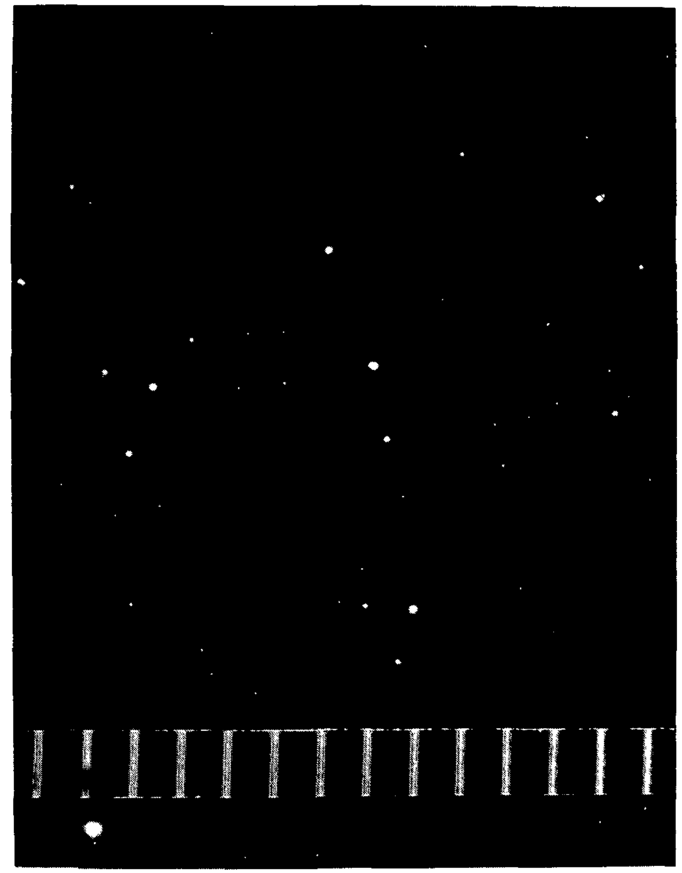

Figure 2

RAW DATA FOR EXPOSED PLATES								CONTROL PLATES	
HOURS AT 125°C	SAMPLE	PLATE COUNT	AVERAGE	HOURS AT 125°C	SAMPLE	PLATE COUNT	AVERAGE	PLATE NO.	PLATE COUNT
1	A	1	1.0	5	A	1	1.3	1	0
	B	0			B	2		2	2
	C	2			C	1		3	5
2	A	2	1.3	6	A	0	0.7	4	4
	B	0			B	2		5	4
	C	2			C	0		6	4
3	A	3	2.0	7	A	0	0.7		
	B	1			B	2		AVERAGE = 3.17	
	C	2			C	0			
4	A	1	1.0	8	A	0	0.0		
	B	1			B	0			
	C	1			C	0			

FIGURE 3

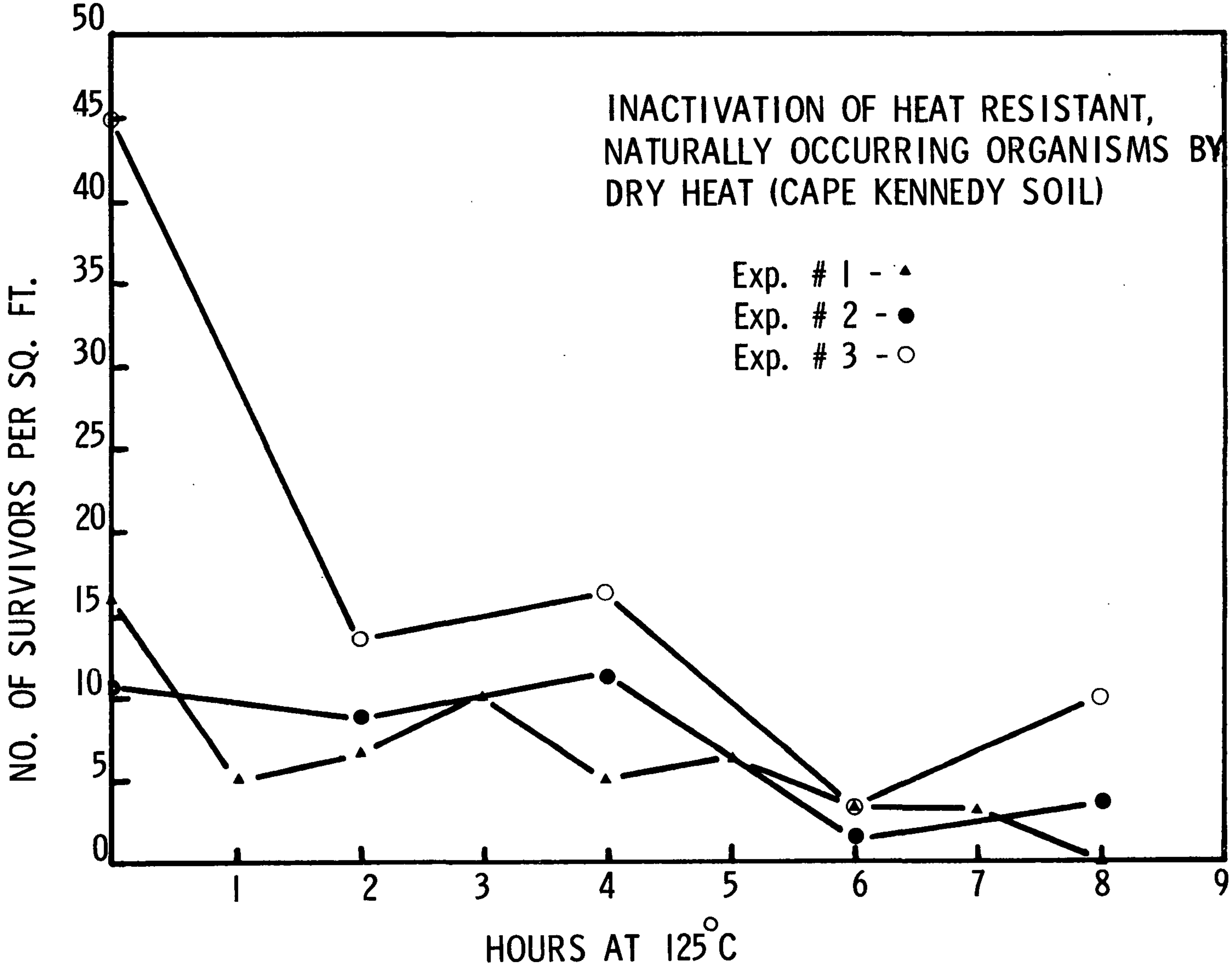
INACTIVATION OF HEAT RESISTANT,
NATURALLY OCCURRING ORGANISMS BY
DRY HEAT (CAPE KENNEDY SOIL)
Exp. # 1 - ▲
Exp. # 2 - ●
Exp. # 3 - ○
NO. OF SURVIVORS PER SQ. FT.
0
5
10
15
20
25
30
35
40
45
50
1
2
3
4
5
6
7
8
9
HOURS AT 125°C

FIGURE 4

Conclusions

Regarding the separation of organisms from soil particles, Freon TF is a satisfactory medium for this purpose. Microscopic observation indicates good dissociation although there appears to be some reagglomeration during the initial settling portion of the washing process when the chance collision of particles is greatest. The low viscosity of Freon TF was a definite aid in filtering the supernatant and concentrating the organisms.

From the results of these experiments, it is apparent that naturally occurring soil organisms can be found in spacecraft assembly and test areas. Further, a very small sub-population shows a high degree of heat resistance compared to the NASA standard test organisms. And finally, it is evident that this heat resistance is a natural characteristic of this sub-population rather than being artificially induced through the mechanism of clumping. This is substantiated by the fact that the particles in these experiments were declumped to a greater degree than might normally be found on spacecraft surfaces.

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